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(54) Title: INTERLEUKIN-2 SIGNAL TRANSDUCERS AND BINDING ASSAYS

(57) Abstract

The invention provides methods and compositions relating to interleukin-2 signal transducers, particularly an isolated human signal transducer and activator of transcription 5 (hStat 5), or a fragment thereof having an hStat 5-specific binding affinity, nucleic acids encoding hStat 5, which nucleic acids may be part of hStat 5-expression vectors and may be incorporated into a recombinant cell, agents which selectively bind hStat 5 or hStat 5 intracellular binding targets, or disrupt the binding of hStat 5 to such intracellular targets, methods of making such agents and hStat 5-specific binding targets in the form of cell surface proteins and nucleic acids. An hStat 5 drug screening assay involves forming mixtures of an hStat 5, an intracellular hStat 5 binding target, and a prospective agent at different concentrations. The mixtures are incubated to permit the binding of the intracellular hStat 5 binding target to the hStat 5 and the mixtures are then analyzed for the presence of such binding. A difference in such binding between the first and second mixtures indicates that the agent is capable of modulating the binding of hStat 5 to an intracellular hStat 5 binding target.

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Interleukin-2 Signal Transducers and Binding Assays

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. 08/235,503, filed April 29, 1994, entitled Transcription Factor-DNA binding Assay, the disclosure of which is hereby incorporated by reference.

INTRODUCTION

Field of the Invention

The field of this invention is human interleukin-2 signal transducers.

Background

Identifying and developing new pharmaceuticals is a multibillion dollar industry in the U.S. alone. Gene specific transcription factors provide a promising class of targets for novel therapeutics directed to these and other human diseases. Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. Methods amenable to automated, cost-effective, high throughput drug screening have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Immunosuppression is therapeutically desirable in a wide variety of circumstances including transplantation, allergy and other forms of hypersensitivity, autoimmunity, etc. Cyclosporin, a widely used drug for effecting immunosuppression, is believed to act by inhibiting a calcineurin, a phosphatase which activates certain transcription factors. However, because of side effects and

toxicity, clinical indications of cyclosporin (and the more recently developed FK506) are limited.

Interleukin-2 (IL-2) is an immunomodulatory cytokine secreted by activated T lymphocytes. IL-2 stimulates the proliferation and differentiated function of hematopoietic cells including T cells, B cells, NK cells, LAK cells, monocytes, macrophages and oligodendrocytes. Specifically, IL-2 is the major autocrine growth factor for T lymphocytes regulating the magnitude of T cell-dependent immune responses; IL-2 stimulates the growth of NK cells and enhances their cytolytic function; and IL-2 stimulates growth and antibody synthesis in B cells. As such, IL-2 signal transduction provides an important target for pharmaceutical intervention in the immune system. Accordingly, it is desired to identify agents which specifically interfere with transduction of IL-2 signalling. Unfortunately, the reagents necessary for the development of high-throughput screening assays for such therapeutics are unavailable.

Relevant Literature

The subunit structure of the IL-2 receptor is described in Cosman et al., 1984; Leonard et al., 1984; Nikaido et al., 1984; Hatakeyama et al., 1989; Takeshita et al., 1992; Voss et al., 1993. The IL-2Ra chain appears to be uniquely dedicated to IL-2 response, whereas the IL-2Rb and IL-2Rg chains are commonly used by other cytokine receptors (Kondo et al., 1993; Noguchi et al., 1993; Giri et al., 1994). On its own, IL-2Ra is capable of low affinity binding to IL-2. High affinity binding and biological response to IL-2 requires, however, association of IL-2Ra with the IL-2Rb and IL-2Rg chains of the receptor (Minamoto et al., 1990; Takeshita et al., 1990). Moreover, functional signaling via the IL-2 receptor is dependent upon the integrity of the intracellular domains of IL-2Rb and IL-2Rg (Kawahara et al., 1994; Nakamura et al., 1994; Nelson et al., 1994). Upon stimulation by IL-2, receptor-bearing cells have been shown to activate the Jak 1 and Jak 3 tyrosine kinases (Boussiotis et al., 1994; Miyazaki et al., 1994; Russell et al., 1994). IL-2 has also been observed to activate an otherwise latent DNA binding activity bearing properties related to Stat proteins (Gilmour and Reich, 1994; Beadling et al., 1994).

Wakao, et al. (1994) disclose a sheep protein, mammary gland factor (MGF) with sequence similarity to hStat 5.

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to interleukin-2 signal transducers. In one embodiment, the invention provides isolated human signal transducer and activator of transcription 5 (hStat 5), or a fragment thereof having an hStat 5-specific binding affinity. The invention provides nucleic acids encoding the subject hStat 5 and hStat 5 fragments, which nucleic acids may be part of hStat 5-expression vectors and may be incorporated into a recombinant cell. The invention provides agents which selectively bind hStat 5 or hStat 5 intracellular binding targets, or disrupt the binding of hStat 5 to such intracellular targets, and methods of making such agents. The invention also provides specific hStat 5 binding targets in the form of cell surface proteins and nucleic acids.

The subject hStat 5 and hStat 5 fragments and find particular use in screening assays for agents or lead compounds for agents useful in the diagnosis, prognosis or treatment of disease, particularly disease associated with undesirable cell growth, differentiation and/or cytokine signal responsiveness. One such assay involves forming mixtures of an hStat 5, an intracellular hStat 5 binding target, and a prospective agent at different concentrations. Typically, one mixture is a negative control (i.e. the agent concentration is zero). The mixtures are incubated to permit the binding of the intracellular hStat 5 binding target to the hStat 5 and the mixtures are then analyzed for the presence of such binding. A difference in such binding between the first and second mixtures indicates that the agent is capable of modulating the binding of hStat 5 to an intracellular hStat 5 binding target.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods and compositions relating to human interleukin-2 signal transducers including hStat 5. A cDNA encoding hStat 5 and its translation product are shown in SEQUENCE ID NOS:1 and 2, respectively.

The subject hStat 5 fragments have one or more hStat 5-specific binding affinities which distinguish other Stats and Stat-related proteins such as MGF,

including the ability to specifically bind at least one natural human intracellular hStat 5-specific binding target or a hStat 5-specific binding agent such as a hStat 5-specific antibody or a hStat 5-specific binding agent identified in assays such as described below. Accordingly, the specificity of hStat 5 fragment specific binding agents is confirmed by ensuring non-crossreactivity with other stats. Furthermore, preferred hStat 5 fragments are capable of eliciting an antibody capable of distinguishing hStat 5 from other Stats and MGF. Methods for making immunogenic peptides through the use of conjugates, adjuvants, etc. and methods for eliciting antibodies, e.g. immunizing rabbits, are well known.

Exemplary natural intracellular binding targets include nucleic acids which comprise one or more hStat 5 DNA binding sites such as the interleukin response element of the gene encoding FcγRI, cell surface proteins such as the hStat 5 binding domain the IL-2 receptor and phosphotryrosine peptide fragments thereof, protein kinases such as Janus tyrosine kinases, transcription factors such as those comprising the transcription initiation complex, etc., and fragments of such targets which are capable of hStat 5-specific binding. Other natural hStat 5 binding targets are readily identified by screening cells, membranes and cellular extracts and fractions with the disclosed materials and methods and by other methods known in the art. For example, two-hybrid screening using hStat 5 fragments are used to identify intracellular targets which specifically bind such fragments. Preferred hStat 5 fragments retain the ability to specifically bind at least one of an hStat 5 DNA binding site and an intracellular domain of an IL-2 receptor subunit. For example, using a strategy analagous to that described in Hou et al. (1994) Science 265: 1701-1706 phosphotyrosine peptides proximal to the carboxyl termixus of the IL-2Rβ chain are shown to inhibit hStat5 DNA binding. IL-2Rβ variants lacking these two peptides are found to lose the ability to activate Stat proteins. Convenient ways to verify the ability of a given hStat 5 fragment to specifically bind such targets include in vitro labelled binding assays such as described below, and EMSAs.

A wide variety of molecular and biochemical methods are available for generating and expressing hStat 5 fragments, see e.g. Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), Current Protocols in Molecular Biology (Eds. Aufubel, Brent, Kingston,

More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1992) or that are otherwise known in the art. For example, hStat 5 or fragments thereof may be obtained by chemical synthesis, expression in bacteria such as E. coli and eukaryotes such as yeast or vaccinia or baculovirus-based expression systems, etc., depending on the size, nature and quantity of the hStat 5 or fragment. The subject hStat 5 fragments are of length sufficient to provide a novel peptide. As used herein, such peptides are at least 5, usually at least about 6, more usually at least about 8, most usually at least about 10 amino acids. hStat 5 fragments may be present in a free state or bound to other components such as blocking groups to chemically insulate reactive groups (e.g. amines, carboxyls, etc.) of the peptide, fusion peptides or polypeptides (i.e. the peptide may be present as a portion of a larger polypeptide), etc.

The subject hStat 5 fragments maintain binding affinity of not less than six, preferably not less than four, more preferably not less than two orders of magnitude less than the binding equilibrium constant of a full-length native hStat 5 to the binding target under similar conditions. Particular hStat 5 fragments or deletion mutants are shown to function in a dominant-negative fashion. Particular hStat 5 fragments containing tyrosine residue 694 are also shown to prevent tyrosine phosphorylation of hStat 5 thereby inhibiting hStat 5 activity. Such fragments provide therapeutic agents, e.g. when delivered by intracellular immunization - transfection of susceptible cells with nucleic acids encoding such mutants.

The claimed hStat 5 and hStat 5 fragments are isolated, partially pure or pure and are typically recombinantly produced. As used herein, an "isolated" peptide is unaccompanied by at least some of the material with which it is associated in its natural state and constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of the total protein (including peptide) in a given sample; a partially pure peptide constitutes at least about 10% , preferably at least about 30%, and more preferably at least about 60% by weight of the total protein in a given sample; and a pure peptide constitutes at least about 70% , preferably at least about 90%, and more preferably at least about 95% by weight of the total protein in a given sample.

The invention provides hStat 5-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, hStat 5-specific agents are useful in a variety of diagnostic applications, especially where disease or disease prognosis is associated with immune disfunction resulting from improper expression of hStat 5. Novel hStat 5-specific binding agents include hStat 5-specific antibodies; novel nucleic acids with sequence similarity to that of the FcγRI receptor promoter as described below; isolated IL-2 receptor subunit domains; other natural intracellular binding agents identified with assays such as one- and two-hybrid screens; non-natural intracellular binding agents identified in screens of chemical libraries, etc.

Generally, hStat 5-specificity of the binding target is shown by binding equilibrium constants. Such targets are capable of selectively binding a hStat 5, i.e. with an equilibrium constant at least about 10^4 M^{-1}, preferably at least about 10^6 M^{-1}, more preferably at least about 10^8 M^{-1}. A wide variety of cell-based and cell-free assays may be used to demonstrate hStat 5-specific binding. Cell based assays include one and two-hybrid screens, mediating or competitively inhibiting hStat 5-mediated transcription, etc. Preferred are rapid in vitro, cell-free assays such as mediating or inhibiting hStat 5-protein (e.g. hStat 5-IL-2 receptor subunit binding), hStat 5-nucleic acid binding, immunoassays, etc. Other useful screening assays for hStat 5/hStat 5 fragment-target binding include fluorescence resonance energy transfer (FRET), electrophoretic mobility shift analysis (EMSA), etc.

The invention also provides nucleic acids encoding the subject hStat 5 and hStat 5 fragments, which nucleic acids may be part of hStat 5-expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for disease associated with expression of a hStat 5), etc. In addition, the invention provides nucleic acids sharing substantial sequence similarity with that of one or more wild-type hStat 5 nucleic acids. Substantially identical or homologous nucleic acid sequences hybridize to their respective complements under high stringency conditions, for example, at 55°C and hybridization buffer comprising 50% formamide in 0.9 M saline/0.09 M sodium citrate (SSC) buffer and remain

bound when subject to washing at 55°C with the SSC/formamide buffer. Where the sequences diverge, the differences are preferably silent, i.e.or a nucleotide change providing a redundant codon, or conservative, i.e. a nucleotide change providing a conservative amino acid substitution.

The subject nucleic acids find a wide variety of applications including use as hybridization probes, PCR primers, therapeutic nucleic acids, etc. for use in detecting the presence of hStat 5 genes and gene transcripts, for detecting or amplifying nucleic acids with substantial sequence similarity such as hStat 5 homologs and structural analogs, and for gene therapy applications. Given the subject probes, materials and methods for probing CDNA and genetic libraries and recovering homologs are known in the art. Preferred libraries are derived from human immune cells, especially CDNA libraries from differentiated and activated human lymphoid cells. In one application, the subject nucleic acids find use as hybridization probes for identifying hStat 5 cDNA homologs with substantial sequence similarity. These homologs in turn provide additional Stats and Stat fragment for use in binding assays and therapy as described herein. hStat 5 encoding nucleic acids also find applications in gene therapy. For example, nucleic acids encoding dominant-negative hStat 5 mutants are cloned into a virus and the virus used to transfect and confer disease resistance to the transfected cells..

Therapeutic hStat 5 nucleic acids are used to modulate, usually reduce, cellular expression or intracellular concentration or availability of active hStat 5. These nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed hStat 5 nucleic acids. Antisense modulation of hStat 5 expression may employ hStat 5 antisense nucleic acids operably linked to gene regulatory sequences. Cell are transfected with a vector comprising an hStat 5 sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous hStat 5 encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a hStat 5 or hStat 5 fragment may be administered to the target cell, in or temporarily isolated from a host, at a

concentration that results in a substantial reduction in hStat 5 expression. For gene therapy involving the transfusion of hStat 5 transfected cells, administration will depend on a number of variables that are ascertained empirically. For example, the number of cells will vary depending on the stability of the transfused cells. Transfusion media is typically a buffered saline solution or other pharmacologically acceptable solution. Similarly the amount of other administered compositions, e.g. transfected nucleic acid, protein, etc., will depend on the manner of administration, purpose of the therapy, and the like.

The subject nucleic acids are often recombinant, meaning they comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome. An isolated nucleic acid constitutes at least about 0.5% , preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

The invention provides efficient methods of identifying pharmacological agents or drugs which are active at the level of hStat 5 modulatable cellular function, particularly hStat 5 mediated interleukin signal transduction. Generally, these screening methods involve assaying for compounds which interfere with hStat 5 activity such as hStat 5-IL-2 receptor binding, hStat 5-DNA binding, etc. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Target therapeutic indications are limited only in that the target cellular function (e.g. gene expression) be subject to modulation, usually inhibition, by disruption of the formation of a complex (e.g. transcription complex) comprising a hStat 5 or hStat 5 fragment and one or more natural hStat 5 intracellular binding targets. Since a wide variety of genes are subject to hStat 5 regulated gene transcription, target indications may include viral, bacterial and fungal

infections, metabolic disease, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation, hypersensitivity, etc. Frequently, the target indication is related to either immune dysfunction or selective immune suppression.

A wide variety of assays for binding agents are provided including labelled in vitro protein-protein and protein-DNA binding assay, electrophoretic mobility shift assays, immunoassays for protein binding or transcription complex formation, cell based assays such as one, two and three hybrid screens, expression assays such as transcription assays, etc. For example, three-hybrid screens are used to rapidly examine the effect of transfected nucleic acids, which may, for example, encode combinatorial peptide libraries or antisense molecules, on the intracellular binding of hStat 5 or hStat 5 fragments to intracellular hStat 5 targets. Convenient reagents for such assays (e.g. GAL4 fusion partners) are known in the art.

hStat 5 or hStat 5 fragments used in the methods are usually added in an isolated, partially pure or pure form and are typically recombinantly produced. The hStat 5 or fragment may be part of a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, sequence-specific nucleic acid binding or stability under assay conditions (e.g. a tag for detection or anchoring).

The assay mixtures comprise at least a portion of a natural intracellular hStat 5 binding target such as an IL-2 receptor subunit domain or a nucleic acid comprising a sequence which shares sufficient sequence similarity with a gene or gene regulatory region to which the native hStat 5 naturally binds to provide sequence-specific binding of the hStat 5 or hStat 5 fragment. Such a nucleic acid may further comprise one or more sequences which facilitate the binding of a second transcription factor or fragment thereof which cooperatively binds the nucleic acid with the hStat 5 (i.e. at least one increases the affinity or specificity of the DNA binding of the other). While native binding targets may be used, it is frequently preferred to use portions (e.g. peptides, nucleic acid fragments) or analogs (i.e. agents which mimic the hStat 5 binding properties of the natural binding target for the purposes of the assay) thereof so long as the portion provides binding affinity and avidity to the hStat 5 conveniently measurable in

the assay. Binding sequences for other transcription factors may be found in sources such as the Transcription Factor Database of the National Center for Biotechnology Information at the National Library for Medicine, in Faisst and Meyer (1991) Nucleic Acids Research 20, 3-26, and others known to those skilled in this art.

Where used, the nucleic acid portion bound by the peptide(s) may be continuous or segmented and is usually linear and double-stranded DNA, though circular plasmids or other nucleic acids or structural analogs may be substituted so long as hStat 5 sequence-specific binding is retained. In some applications, supercoiled DNA provides optimal sequence-specific binding and is preferred. The nucleic acid may be of any length amenable to the assay conditions and requirements. Typically the nucleic acid is between 8 bp and 5 kb, preferably between about 12 bp and 1 kb, more preferably between about 18 bp and 250 bp, most preferably between about 27 and 50 bp. Additional nucleotides may be used to provide structure which enhances or decreased binding or stability, etc. For example, combinatorial DNA binding can be effected by including two or more DNA binding sites for different or the same transcription factor on the oligonucleotide. This allows for the study of cooperative or synergistic DNA binding of two or more factors. In addition, the nucleic acid can comprise a cassette into which transcription factor binding sites are conveniently spliced for use in the subject assays.

The assay mixture also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than about 2,500, preferably less than about 1000, more preferably, less than about 500. Candidate agents comprise functional chemical groups necessary for structural interactions with proteins and/or DNA, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups, more preferably at least

three. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the foremention functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof, and the like. Where the agent is or is encoded by a transfected nucleic acid, said nucleic acid is typically DNA or RNA.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the hStat 5 specifically binds the cellular binding target, portion or analog. The mixture components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4 and 40 °C, more commonly between 15 and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between .1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of specific binding between the hStat 5 and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate which may be any solid from which the unbound components may be conveniently separated. The solid substrate may be made of a wide variety of materials and in a wide variety of shapes, e.g. microtiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, primarily to minimize background binding, for ease of washing and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting reservoir such as a microtiter plate well, rinsing a bead (e.g. beads with iron cores may be readily isolated and washed using magnets), particle, chromatographic column or filter with a wash solution or solvent. Typically, the separation step will include an extended rinse or wash or a plurality of rinses or washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc. may exploit a polypeptide specific binding reagent such as an antibody or receptor specific to a ligand of the polypeptide.

Detection may be effected in any convenient way. For cell based assays such as one, two, and three hybrid screens, the transcript resulting from hStat 5-target binding usually encodes a directly or indirectly detectable product (e.g. galactosidase activity, luciferase activity, etc.). For cell-free binding assays, one of the components usually comprises or is coupled to a label. A wide variety of labels may be employed - essentially any label that provides for detection of bound protein. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to the protein e.g. a phosphate group comprising a radioactive isotope of phosphorous, or

incorporated into the protein structure, e.g. a methionine residue comprising a radioactive isotope of sulfur.

A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters. The methods are particularly suited to automated high throughput drug screening. Candidate agents shown to inhibit hStat 5 - target binding or transcription complex formation provide valuable reagents to the pharmaceutical industries for animal and human trials.

As previously described, the methods are particularly suited to automated high throughput drug screening. In a particular embodiment, the arm retrieves and transfers a microtiter plate to a liquid dispensing station where measured aliquots of each an incubation buffer and a solution comprising one or more candidate agents are deposited into each designated well. The arm then retrieves and transfers to and deposits in designated wells a measured aliquot of a solution comprising a labeled transcription factor protein. After a first incubation period, the liquid dispensing station deposits in each designated well a measured aliquot of a biotinylated nucleic acid solution. The first and/or following second incubation may optionally occur after the arm transfers the plate to a shaker station. After a second incubation period, the arm transfers the microtiter plate to a wash station where the unbound contents of each well is aspirated and then the well repeatedly filled with a wash buffer and aspirated. Where the bound label is radioactive phosphorous, the arm retrieves and transfers the plate to the liquid dispensing station where a measured aliquot of a scintillation cocktail is deposited in each designated well. Thereafter, the amount of label retained in each designated well is quantified.

In more preferred embodiments, the liquid dispensing station and arm are capable of depositing aliquots in at least eight wells simultaneously and the wash station is capable of filling and aspirating ninety-six wells simultaneously. Preferred robots are capable of processing at least 640 and preferably at least about 1,280 candidate agents every 24 hours, e.g. in microtiter plates. Of course, useful agents are identified with a range of other assays (e.g. gel shifts, etc.) employing the subject hStat 5 and hStat 5 fragments.

The subject hStat 5 and hStat 5 fragments and nucleic acids provide a wide variety of uses in addition to the in vitro binding assays described above. For example, cell-based assays are provided which involve transfecting an IL-2 receptor subunit or functional fragment thereof expressing cell with an hStat 5 inducible reporter such as luciferase. Agents which modulate hStat 5 mediated cell function are then detected through a change in the reporter. Another approach is a transient expression assay. In this method, cells are transfected with one or more constructs encoding in sum, a polypeptide comprising a portion of hStat 5 capable of selectively binding an natural IL-2 receptor target and a reporter under the transcriptional control of a promoter comprising a functional hStat 5 binding site. The cell may advantageously also be cotransfected with a construct encoding an hStat 5 activator, usually a tyrosine kinase, particularly a Jak kinase.

The subject compositions also provide therapeutic applications. For example, hStat 5 peptides comprising tyrosine residue 694 or IL-2 receptor peptides capable of selectively binding said hStat 5 peptides find use in treating disease associated with undesirable cell growth, differentiation, particularly immune cell differentiation, and cytokine, particularly interleukin, more particularly IL-2, responsiveness. For therapeutic uses, the compositions and agents disclosed herein may be administered by any convenient way, preferably parenterally, conveniently in a physiologically acceptable carrier, e.g., phosphate buffered saline, saline, deionized water, or the like. Typically, the compositions are added to a retained physiological fluid such as blood or synovial fluid. Generally, the amount administered will be empirically determined, typically in the range of about 10 to 1000 μg/kg of the recipient. For peptide agents, the concentration of will generally be in the range of about 100 to 500 μg/ml in the

dose administered. Other additives may be included, such as stabilizers, bactericides, etc. These additives will be present in conventional amounts.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Peripheral blood lymphocytes (PBLs) isolated from human plasma were cultured in the presence (hereafter designated "stimulated") or absence (hereafter designated "resting") of PHA for three days. PHA stimulated cells were washed and cultured for an additional 12 hours in the absence of PHA. Both cultures were exposed to varying concentrations of recombinant IL-2 for 15 minutes then used to prepare nuclear extracts (Experimental Procedures). Gel mobility shift assays were performed using a DNA probe derived from the promoter of the gene encoding the FcgRI immunoglobulin receptor that is known to bind Stat proteins avidly (Kotanides and Reich, 1993).

Nuclear extracts prepared from both PHA-stimulated and resting PBLs contained three DNA binding activities capable of forming stable complexes with the FcgRI probe. The slowest migrating complex, designated P1, was eliminated when challenged with specific competitor DNA, yet was insensitive to competition by a mutated derivative of the FcgRI probe (Experimental Procedures). The two more rapidly migrating complexes were insensitive to challenged with specific competitor DNA and therefore deemed non-specific (NS).

IL-2 treatment of both PHA-stimulated and resting PBL cultures failed to substantially alter the abundance of the activities responsible for generating either the P1 or NS complexes. Cytokine treatment did, however, lead to the appearance of a DNA binding activity, designated P2, that was sensitive to competition by the FcgRI probe. Optimal activation of the P2 complex in resting PBLs required 30 ng/ml IL-2. PHA stimulated PBLs contained peak levels of this activity when stimulated at the lowest dose of IL-2 tested (3 ng/ml).

A transformed human lymphocyte cell line termed YT (Yodoi et al., 1985) was identified as a potential source for purification of IL-2 induced transcription factors. Nuclear extracts prepared from uninduced YT cells were observed to

contain several activities capable of binding to the FcgRI probe. Competition assays revealed the slowest migrating complex (Y1) to be specific and the more rapidly migrating complexes to be non-specific (NS). None of the three activities appeared to change as a function of IL-2 presentation. Following exposure for 15 minutes to recombinant IL-2, however, two new DNA binding activities were observed. One activity, designated Y2, was induced in response to intermediate concentrations of IL-2 (10-30 ng/ml). The other, designated Y3, required exposure to higher concentration of cytokine (30-100 ng/ml). Both of the IL-2 induced DNA binding activities obtained from YT cells were sensitive to specific competitor DNA, yet insensitive to the mutated derivative of the FcgRI probe. Two observations indicated that the constitutive (P1) and IL-2 induced (P2) complexes obtained from PBLs might correspond, respectively, to the IL-2 induced complexes obtained from YT cells (Y2 and Y3). First, they co-migrated when analyzed by the gel mobility shift assay (P1 = Y3 and P2 = Y2). Second, the P2 and Y2 complexes were induced in resting PBLs and YT cells with similar dose dependencies (10-30 ng/ml).

Having obtained provisional evidence that PBLs and YT cells induce similar DNA binding activities in response to IL-2 we set out to purify the polypeptides specifying these activities. Nuclear extracts were prepared from 40 liters of YT cells that had been exposed for 15 minutes to 30 ng/ml of recombinant IL-2. A purification scheme involving ammonium sulfate precipitation followed by S-Sepharose, DNA affinity and Q-Sepharose chromatography (Experimental Procedures) led to the isolation of a complex group of polypeptides that migrated on denaturing polyacrylamide gels in the range of 85-100 kD. Western blotting assays using anti-phosphotyrosine antibodies gave evidence that many, if not all, of these proteins were tyrosine phosphorylated.

The proteins purified according to procedures outlined in the preceding paragraph were cleaved with lysine-C and resulting peptide fragments were fractionated by reversed phase capillary high-performance liquid chromatography (HPLC). Partial amino acid sequences were resolved for six peptides (Experimental Procedures). When compared with a data base of known protein

sequences, two of the sequenced peptides were found to correspond to Stat 1 (Schindler et al., 1992) and four to Stat 3 (Akira et al., 1994).

Apparently, YT cells induced with IL-2 contain activated Stat 1 and Stat 3. Recall, however, that at least three distinct Stat-like complexes were observed in YT nuclear extracts. One such complex, termed Y1, was present irrespective of IL-2 presentation. The other two, designated Y2 and Y3, were induced, respectively, by intermediate and relatively high levels of recombinant IL-2.

In order to determine the molecular identities of the three complexes, antibodies specific to Stat 1 and Stat 3 were incubated with nuclear extracts derived from uninduced and IL-2 induced YT cells. Antibodies specific to Stat 1 did not affect the Y1 complex formed between uninduced YT nuclear extract and the FcgRI probe. However, when incubated with extracts prepared from cells exposed to 30 ng/ml IL-2, the Stat-1 antibody altered the mobility of the most rapidly migrating, IL-2 induced complex (Y3). Compared with untreated extracts, or extracts incubated with control antibodies, those incubated with antibodies to Stat 1 resulted in a protein:DNA complex that migrated at a significantly retarded (supershifted) rate. Identical results were observed with nuclear extracts prepared from PBLs induced with 30 ng/ml IL-2. That is, the P1 complex was selectively supershifted by Stat 1 antibodies. We therefore conclude that Stat 1 was activated in YT cells in response to relatively high concentrations of IL-2. PBLs also contain Stat 1 that, at least in the case of the PHA-stimulation, was marginally induced by IL-2.

Antibodies to Stat 3 were also tested using nuclear extracts prepared from uninduced YT cells, as well as YT cells and PBLs that had been exposed for 15 minutes to 30 ng/ml IL-2. In this case we observed a distinctive alteration in the migration of the Y1 complex formed by YT nuclear extracts, irrespective of exposure to IL-2. Antibodies to Stat 3 did not affect the abundance or migration of DNA binding activities derived from IL-2 induced PBLs, irrespective of stimulation by PHA. According to these observations, we conclude that active Stat 3 is constitutively present in YT cells and is not further stimulated by IL-2 in either YT cells or PBLs.

The observations outlined thus far provide a molecular link between Stat 1 and a protein:DNA complex induced in YT cells by relatively high levels of IL-

2 (designated Y3), as well as link between Stat 3 and a complex present in uninduced YT cells (designated Y1). Left unresolved, however, was the molecular nature of the Y2 activity induced in YT cells by intermediate concentrations of IL-2. A co-migrating DNA:protein complex, designated P2, was also induced by intermediate concentrations of IL-2 in resting PBLs, and by very low concentrations of IL-2 in PHA stimulated PBLs. In order to resolve the identity of the polypeptides specifying this activity, the complex of Stat proteins purified from YT cells was purged of Stat 1 and Stat 3 by immunodepletion.

Protein purified from 20 liters of IL-2 induced YT cells was incubated sequentially with antibodies specific to Stat 1 and Stat 3 and Sepharose beads linked to protein-A (Experimental Procedures). Load, flow-through, wash and bound fractions were then analyzed by SDS gel electrophoresis and subsequent staining using Coomassie blue as well as antibodies to phosphotyrosine, Stat 1 and Stat 3. The resulting staining patterns revealed effective depletion of Stat 1 and Stat 3. Both Coomassie and anti-phosphotyrosine staining indicated, however, that the flow through fraction retained Stat-like proteins. The three most prominent polypeptides remaining following immunodepletion were excised and individually digested with lysine-C. Resulting peptide fragments were separated by reversed phase capillary HPLC and subjected to partial amino acid sequence analysis.

The largest of the three polypeptide bands, which migrated with an apparent molecular mass of 98 Kd, yielded three peptide sequences, all of which matched the sequence of a previously characterized protein variously termed mammary gland factor (MGF) or Stat 5 (Wakao et al., 1994). Two of the peptides yielded unambiguous sequences of 10 and 14 residues. Both of these sequences were identical to segments of MGF/Stat 5. The third peptide, designated peak #12, did not contain an identifiable amino acid residue in the fifth Edman degradation cycle. Ten of the eleven residues of this sequence did, however, match perfectly with a segment located close to the carboxyl terminus of MGF/Stat 5. The unidentified residue of this peptide corresponded to tyrosine residue 694 of MGF/Stat 5, which has been identified as the substrate for Jak-mediated phosphorylation (Gouilleux et al., 1994).

Reasoning that the ambiguity observed upon amino acid sequence analysis of this third peptide might reflect the fact the fifth residue was phosphorylated on tyrosine, and therefore not extracted by the non-polar solvent used to extract the phenylthiohydantion during Edman degradation (Aebersold et al., 1991; Yip and Hutchens, 1992), we performed matrix-assisted laser ionization desorption/ionization (MALDI) mass spectrometry analysis. The observed mass of the peptide (1,297.0 +/- 2 daltons) corresponded very closely to that predicted for the tyrosine phosphoform of the sequence AVDGYVKPQIK (SEQUENCE ID NO: 2, residues 690-700) (1,298.4 daltons). Given that the affinity chromatographic step of our purification procedure selects for the active form of Stat proteins (Hou et al., 1994), and that the Stat activation cycle entails tyrosine phosphorylation (Darnell et al., 1994), it is not surprising that the Stat protein activated in IL-2 induced YT cells is tyrosine phosphorylated.

Peptide sequence analysis of two smaller Stat-like proteins remaining after immunodepletion (migrating with apparent masses of 94 and 88 Kd) also yielded sequences identical to MGF/Stat 5. We thus conclude that the sample of proteins purified from IL-2 induced YT cells, following depletion of Stat 1 and Stat-3, is largely composed of polypeptides related to MGF/Stat 5. Moreover, the three predominant species (98, 94 and 88 Kd) all appear to be tyrosine phosphorylated on the same residue. We conclude that activation of this family of proteins by IL-2 in human YT cells entails tyrosine phosphorylation at the same position as prolactin-mediated activation of MGF/Stat 5 (Gouilleux et al., 1994).

The relevance of the aforementioned finding may be signified by the fact that prolactin and IL-2 activate different Jak kinases. Two recent studies have provided evidence that prolactin activates Jak 2 (Gilmour and Reich, 1994; Rui et al., 1994). In contrast, IL-2 has been shown to activate Jak 1 and Jak 3 (Boussiotis et al., 1994; Miyazaki et al., 1994; Russell et al., 1994). Having found that MGF and hStat 5 are tyrosine phosphorylated on the same residue (694), we conclude that the specificity of activation is not controlled by Jak kinases, but rather that the functional Jak employed in Stat activation is dictated by the intracellular domain of the relevant receptor. In the case of the prolactin receptor, MGF activation is mediated by Jak 2 due, presumably, to the affinity of Jak 2 to the intracellular domain of the prolactin receptor. In contrast, hStat 5 is

activated by Jak 1 and/or Jak 3 by virtue of their affinity to one or more chains of the IL-2 receptor. Hence, we conclude that specificity of Stat activation rests on coupling of the latent transcription factor to the intracellular domain of its cognate receptors (Greenlund et al., 1994; Hou et al., 1994).

Having established a molecular link between Stat 5 and the DNA binding activity induced by IL-2 in YT cells (designated Y2), we set out to determine the identity of the DNA binding activity induced by IL-2 in PBLs. Whereas the activity induced by IL-2 in both resting and PHA-stimulated PBLs (designated P2) co-migrated on non-denaturing gels with that induced in YT cells (Y2), we did not have antibodies that would allow rigorous testing of the identity of the Stat protein induced in PBLs. As such, we purified the activity in order to obtain partial amino acid sequence of the relevant polypeptides.

Lymphocytes were isolated from 10 units of human blood and grown for three days in the presence of PHA. The cells were then washed, cultured for 12 hours in the absence of PHA, exposed for 15 minutes to 10ng/ml IL-2, harvested and used to prepare nuclear extracts. The IL-2 induced DNA binding activity was purified by the same method used to isolate Stat proteins from YT cells (Experimental Procedures).

These procedures led to the purification of two polypeptides that migrated with apparent molecular masses of 96 and 95 Kd. Western blot assays provided evidence that both of these polypeptides reacted strongly with antibodies to phosphotyrosine. Each polypeptide was excised, digested with lysine-C and the resulting peptide fragments were fractionated by capillary HPLC. The two proteins yielded very similar lysine-C digests as assessed by the UV spectral traces of the resulting chromatograms. Mass spectrometry analysis of individual, co-eluting peptides provided additional evidence of a close relatedness between the 96 and 95 Kd polypeptides. Two peptides were subjected to gas phase sequencing, yielding sequences highly related to MGF/Stat 5. We thus conclude that the predominant Stat protein induced by IL-2 in PHA-stimulated human lymphocytes is highly related to MGF/Stat 5.

A cDNA clone corresponding to the human form of Stat 5 was isolated and sequenced. Conceptual translation of the DNA sequence specified an open reading frame (ORF) of 794 amino acid residues which, in an unmodified state,

predicts a molecular mass of 90,544 daltons. Segments identical to all three of the peptides derived from the IL-2 induced protein from YT cells (Y2) and one of the peptides derived from the IL-2 induced protein from PBLs (P2) were observed in the ORF. The second peptide sequence derived from the IL-2 induced protein purified from PBLs matched the ORF at five consecutive residues, yet diverged significantly at amino acid residue 687. This may represent the result of alternative splicing or presence of a second, highly related hStat 5 gene.

A comparison of the sequence of the cloned gene product, hereby designated human Stat 5 (hStat 5), and the previously characterized MGF protein of sheep (Wakao et al., 1994) reveals that although significant segments of amino acid sequence identity occur throughout the two proteins, three divergent regions are also observed (residues 90-107, 260-272 and 717-COOH). As such, the sheep and human proteins may be encoded by non-homologous genes.

The tissue distribution of hStat 5 gene expression was examined by Northern blotting. Messenger RNAs of measuring roughly 3, 4 and 6 Kb in length were observed. The larger two mRNAs were observed in all human tissues that were examined, with roughly equivalent levels occurring in placenta, skeletal muscle, spleen, thymus, prostate, testis, ovary, small intestine, colon and PBLs, slightly lower levels in heart, lung, kidney and pancreas, and considerably lover levels in brain and liver. The small (3 Kb) mRNA was observed in a more restricted pattern, limited to placenta, skeletal muscle and testis. It is unclear whether these various hStat 5 mRNAs are expressed as differentially processed products of a single gene or the transcribed products of distinct genes.

Experimental Procedures:

Cell Culture:

The transformed human lymphocyte cell line YT (Yodoi et al., 1985) was grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 10mM HEPES pH 7.2, 10mM b-mercaptoethanol, 2mM L-glutamine, 100mg/ml streptomycin and 100mg/ml ampicillin. Cells grown to a density of 7 X 10^5/ml in T-150 flasks were exposed to recombinant IL-2 (Biosource International) for 15 minutes at 37°C, harvested by centrifugation and used to prepare nuclear extracts (Dignam et al., 1983). Peripheral blood lymphocytes (PBLs) obtained from Irvin Memorial Blood Bank (San Francisco) were harvested by centrifugation,

resuspended in phosphate buffered saline and applied atop Lymphoprep (Nycomed AS) in 50ml conical centrifuge tubes. Following centrifugation the lymphocyte layer was retrieved, washed and resuspended in the same culture medium used for growth of YT cells. Cells were grown either in the presence or absence of *Phaseolus vulgaris* lectin (PHA) (Sigma) for three days. PHA stimulated cells were washed, resuspended in culture medium lacking PHA and grown for 12 hours. IL-2 treatment and preparation of nuclear extracts was the same as used for YT cells.

Purification of IL-2 induced transcription factors:

Nuclear extracts from YT cells (1 g) and PBLs (0.25 g) were precipitated with 30% ammonium sulfate. The proteins were then removed by centrifugation and the supernatant was then treated with 60% ammonium sulfate. Proteins precipitating between 30-60% ammonium sulfate were recovered by centrifugation, resuspended in 20mM Hepes (pH 7.9), 25% (V/V) glycerol, 0.1M NaCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 0.5mM phenylmethylsulfonate, 0.5mM dithiothreitol (DTT), aprotinin at 1mg/ml, pepstatin at 1mg/ml, leupeptin at 1mg/ml, 1mM benzamidine, 1mM sodium vanadate, 1mM NaF, 5mM b-glycerolphosphate (buffer C), and dialyzed against buffer C supplemented with NaCl to a final concentration of 150mM.

After dialysis, insoluble proteins were removed by centrifugation and the remaining material was chromatographed over a 100ml S-Sepharose (Pharmacia) column. Protein flow-through was mixed with a DNA-affinity resin prepared by coupling synthetic, biotinylated DNA corresponding to the IL-4 response element of the gene encoding FcgRI (5'-GTATTTCCCAGAAAAGGAAC-3) to streptavidin agarose (Sigma). After binding (2 hours at 4°C), the affinity matrix was placed on a disposable column and washed sequentially with 10ml of buffer C, 4ml of buffer C supplemented with a mutated variant of the IL-4 response element (5'-GTATCACCAGTCAAGGAAC-3') at 0.2mg/ml, and 10ml of buffer C. Protein was eluted by exposure to buffer C supplemented with 0.35M NaCl, dialyzed against buffer C, and placed on a 0.5ml Q-Sepharose (Pharmacia) column. The column was washed with 5ml of buffer C and protein was eluted with 1ml of buffer C supplemented with 0.35M NaCl.

Partial amino acid sequencing of IL-2 induced transcription factors:

Purified IL-2 induced DNA binding proteins were subjected to SDS-gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was stained with Coomassie blue R-250 in 40% methanol and 0.1% acetic acid for 30 seconds then destained for 5 minutes with 10% acetic acid in 50% methanol. Relevant polypeptides were excised from the membrane and alkylated with isopropylacetamide (Krutsch and Inman, 1993; Henzel et al., 1994) and then digested in 50ul of 0.1M ammonium bicarbonate, 10% acetonitrile with 0.2mg of lysine-C (Wako) at 37°C for 17 hours. The solution was then concentrated to 20ul and directly injected onto a capillary high-performance liquid chromatogram. The HPLC consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 UV detector equipped with a Z-shaped flow cell (LC Packings, Inc.). A 30 cm length of 0.025 mm ID glass capillary was connected to the outlet of the Z-shaped cell inside the detector housing to minimize the delay volume. The total delay volume was 0.45 ml which corresponded to a delay of 6 seconds for a flow rate of 3.5 ml/min. The short delay greatly facilitated hand collection of individual peptide peaks. Peptides were separated on a C18 capillary column (0.32 by 100mm) (LC Packing) developed with 0.1% aqueous trifloracetic acid as buffer A and acetonitrile containing 0.07% trifluoroacetic acid as buffer B . Isolated peptides were sequenced on a 470A Applied Biosystems sequencer. Residual peptides retained on the PVDF membrane were also subjected to gas phase sequencing. In the case of hStat 5 purified from PHA stimulated PBLs, this provided partial amino acid sequence of a very large lysine-C peptide, designated PBL-MB (membrane bound) co-linear with the carboxyl terminus of the intact hStat 5 protein. The same sequence was found for this peptide regardless of whether derived from the 96 or 95 Kd polypeptides (see Figure 5). Sequence interpretation was performed on a DEC 5900 computer (Henzel et al., 1987).

Immunodepletion, supershifting and oligonucleotide competition assays:

Protein:DNA complexes were visualized by a gel mobility shift assay under non-denaturing conditions. Specificity of protein:DNA interaction was tested using 100-fold molar excess of either the native FcgRI probe (5'-GTATTTCCCAGAAAAGGAAC-3', SEQUENCE ID NO: 3) or the mutated derivative (5'-GTATCACCCAGTCAAGGAAC-3', SEQUENCE ID NO: 4).

Antibody supershift experiments were performed by incubating protein samples with antibodies (Santa Cruz Biotech) for 30 minutes at 4°C prior to exposure to the FcgRI DNA probe. Proteins purified from YT cells were purged of Stat 1 and Stat 3 by immunodepletion. 500mg of each antibody (specific to Stat 1 and Stat 3) were incubated for 2 hours at room temperature with a slurry of protein-A Sepharose beads (Pharmacia) sufficient to yield 100ml of bed volumn. Beads were washed three times with 1ml of buffer C and then incubated for 2 hours at room temperature with Stat proteins purified from IL-2 induced YT cells. Beads were recovered by centrifugation and washed three times with 1ml of buffer C. Unbound, wash and bound fractions were recovered and subjected to SDS-gel electrophoresis for subsequent staining with Coomassie blue, anti-phosphotyrosine antibodies and Stat antibodies.

Mass spectrometry:

Aliquots (0.2ml) of the capillary HPLC purified lysine-C peptides were mixed on the sample probe tip with 0.2ml of a-cyano-4-hydroxycinnamic acid (saturated in 50% acitonitrile 2% TFA). MALDI mass spectra were obtained with a Vestec (Houston, TX) LaserTec ResearcH laser desorption linear time-of-flash mass spectrometer equipped with a 337nm VSL-337 ND nitrogen laser (Laser Sciences, Inc).

Cloning of hStat 5 cDNA:

A cDNA library prepared from human umbilical vein endothelial cells was probed at low stringency with a fragment of the IL-4 Stat cDNA (Hou et al., 1994) corresponding to the SH3 and SH2 domains of the protein. Radiolabeled probe DNA was hybridized with filter lifts at 42°C in a solution containing 20% formamide, 10X Denhards, 5X SSPE, 0.5% SDS and 100mg/ml salmon sperm DNA. After hybridization (16 hours) filters were washed twice at 42°C in a 2X SSPE, 0.5% SDS and twice at 42°C in 2X SSPE. Roughly equivalent numbers of clones encoding IL-4 Stat and hStat 5 were recovered. A 3.8 Kb hStat 5 cDNA clone was sequenced on both DNA strands using an Applied Biosystems automated DNA sequencer.

Northern Blotting:

RNA blot hybridization with a uniformly labeled DNA probe prepared from the hStat 5 cDNA clone and Multiple Tissue Northern Blot membranes (Clone-tech) were used. The probe DNA fragment corresponded to the region of the hStat 5 cDNA that encodes the amino terminal segment of the protein (excluding the putative SH3 and SH2 domains). Probe labeling, hybridization and membrane washing were performed as described (Sambrook et al., 1989).

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Aebersold et al., (1991) *Anal. Biochem.* 199:51-60; Akira et al., (1994) *Cell* 77:63-71; Beadling et al., (1994) *EMBO J.* 13:5605-5615; Boussiotis et al., (1994) *Science* 266:1039-1042; Darnell et al., (1994) *Science* 264:1415-1421; Dignam et al., (1983) *Nucl. Acids Res.* 11:1475-99; Cosman et al., (1984) *Nature* 312:768-771; Gilmore et al., (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:6850-6854; Giri et al., (1994) *EMBO J.* 13:2822-2830; Gouilleux et al., (1994) *EMBO J.* 13:4361-4369; Greenlund et al., (1994) *The EMBO J.* 13:1591-1600; Hatakeyama et al., (1989) *Science* 244:551-556; Henzel et al., (1994) *Methods: Companion Methods Enzymol.* 6:239-247; Henzel et al., (1987) *J. Chromatogr.* 404:41-52; Hou et al., (1994) *Science* 265:1701-1706; Kawahara et al., (1994) *Mol. Cell. Biol.* 14:5433-5440; Kelly et al., (1991) *Endocrine Rev.* 12:235-251; Kondo et al., (1993) *Science* 262:1874-1877; Kotanides et al., (1993) *Science* 262:1265-1267; Krutzsch et al., (1993) *Anal. Biochem.* 209:109-116; Leonard et al., (1984) *Nature* 311:626-631; Minamoto et al., (1990) *J. Immunol.* 145:217-2182; Miyazaki et al., (1994) *Science* 266:1045-1047; Nakamura et al., (1994) *Nature* 369:330-333; Nelson et al., (1994) *Nature* 369:333-336; Nikaido et al., (1984) *Nature* 311:631-635; Noguchi et al., (1993) *Science* 262:1877-1880; Rui et al., (1994) *J. Biol. Chem.* 269:5364-5368; Russell et al., (1994) *Science* 266:1042-1045; Sambrook et al., (1989) Molecular Cloning (Cold Spring Harbor: Cold Spring Harbor Laboratory); Schindler et al., (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:7836-7839; Smith (1988) *Science* 240:1169-1176; Takeshita et al., (1992) *Science* 257:379-382; Takeshita et al., (1990) *Int. Immunol.* 2:477-480; Taniguchi et al., (1986) *Immunol. Rev.* 92:121-133; Taniguchi and Minami (1993) *Cell* 73:5-8; Voss et al., (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:2428-2432; Waldmann (1993) *Immunol. Today* 15:19-26; Wakao et al., (1994) *The EMBO J.* 13:2182-2191; Yip and Hutchens (1992) *FEBS Lett.* 308:149-153; and Yodoi et al., (1985) *J. Immun.* 134:1623-1630.

EXAMPLES

1. Protocol for hStat 5 - IL-2 Receptor-peptide binding assay.

A. Reagents:

- Neutralite Avidin: 20 μg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5 % NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- ^{33}P hStat 5 10x stock: 10^{-8} - 10^{-6} M "cold" hStat 5 supplemented with 200,000-250,000 cpm of labeled, inactive and truncated hStat 5 (Beckman counter). Place in the 4 °C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.
- IL-2-receptor-peptides: 10^{-8} - 10^{-5} M of each IL-2 receptor biotinylated peptides in PBS.

B. Preparation of assay plates:

- Coat with 120 μl of stock N-Avidin per well overnight at 4 °C.
- Wash 2X with 200 μl PBS.
- Block with 150 μl of blocking buffer.
- Wash 2X with 200 μl PBS.

C. Assay:

- Add 40 μl assay buffer/well.
- Add 10 μl compound or extract.
- Add 10 μl ^{33}P-hStat 5 (20,000-25,000 cpm/0.1-10 pmoles/well = 10^{-9}- 10^{-7} M final concentration).
- Shake at 25C for 15 min.
- Incubate additional 45 min. at 25C.
- Add 40 μl IL-2 receptor peptide mixture (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hr at RT.
- Stop the reaction by washing 4X with 200 μl PBS.

- Add 150 μl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no receptor peptide added)

b. Soluble (non-biotinylated receptor peptide) at 80% inhibition.

2. Protocol for hStat 5 - DNA binding assay.

A. Reagents:

- Neutralite Avidin: 20 μg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5 % NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- ^{33}P hStat 510x stock: 10^{-6} - 10^{-8} M "cold" hStat 5 supplemented with 200,000-250,000 cpm of labeled hStat 5 (Beckman counter). Place in the 4 °C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.
- Oligonucleotide stock: (specific biotinylated). Biotinylated oligo at 17 pmole/μl, hStat 5 binding site: (BIOTIN)-GTATTTCCCAGAAAAGGAAC (SEQUENCE ID NO: 3)

B. Preparation of assay plates:

- Coat with 120 μl of stock N-Avidin per well overnight at 4 °C.
- Wash 2X with 200 μl PBS.
- Block with 150 μl of blocking buffer.
- Wash 2X with 200 μl PBS.

C. Assay:

- Add 40 μl assay buffer/well.
- Add 10 μl compound or extract.
- Add 10 μl ^{33}P-hStat 5 (20,000-25,000 cpm/0.1-10 pmoles/well = 10^{-9}- 10^{-7} M final concentration).

- Shake at 25C for 15 min.
- Incubate additional 45 min. at 25C.
- Add 40 μl oligo mixture (1.0 pmoles/40 ul in assay buffer with 1 ng of ss-DNA)
- Incubate 1 hr at RT.
- Stop the reaction by washing 4X with 200 μl PBS.
- Add 150 μl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no oligo added)

b. Specific soluble oligo at 80% inhibition.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: TULARIK, INC.

(ii) TITLE OF INVENTION: INTERLEUKIN-2 SIGNAL TRANSDUCERS AND BINDING ASSAYS

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: USA
(F) ZIP: 94111-4187

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US96/
(B) FILING DATE: 1996 FEB 22
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US Serial No. 08/393,333
(B) FILING DATE: 1995 FEB 23
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2385 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2382

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GCG GGC TGG ATC CAG GCC CAG CAG CTG CAG GGA GAC GCG CTG CGC    48
Met Ala Gly Trp Ile Gln Ala Gln Gln Leu Gln Gly Asp Ala Leu Arg
 1               5                  10                  15
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CAG ATG CAG GTG CTG TAC GGC CAG CAC TTC CCC ATC GAG GTC CGG CAC     96
Gln Met Gln Val Leu Tyr Gly Gln His Phe Pro Ile Glu Val Arg His
                20                  25                  30

TAC TTG GCC CAG TGG ATT GAG AGC CAG CCA TGG GAT GCC ATT GAC TTG    144
Tyr Leu Ala Gln Trp Ile Glu Ser Gln Pro Trp Asp Ala Ile Asp Leu
            35                  40                  45

GAC AAT CCC CAG GAC AGA GCC CAA GCC ACC CAG CTC CTG GAG GGC CTG    192
Asp Asn Pro Gln Asp Arg Ala Gln Ala Thr Gln Leu Leu Glu Gly Leu
        50                  55                  60

GTG CAG GAG CTG CAG AAG AAG GCG GAG CAC CAG GTG GGG GAA GAT GGG    240
Val Gln Glu Leu Gln Lys Lys Ala Glu His Gln Val Gly Glu Asp Gly
    65                  70                  75                  80

TTT TTA CTG AAG ATC AAG CTG GGG CAC TAC GCC ACG CAG CTC CAG AAA    288
Phe Leu Leu Lys Ile Lys Leu Gly His Tyr Ala Thr Gln Leu Gln Lys
                85                  90                  95

ACA TAT GAC CGC TGC CCC CTG GAG CTG GTC CGC TGC ATC CGG CAC ATT    336
Thr Tyr Asp Arg Cys Pro Leu Glu Leu Val Arg Cys Ile Arg His Ile
            100                 105                 110

CTG TAC AAT GAA CAG AGG CTG GTC CGA GAA GCC AAC AAT TGC AGC TCT    384
Leu Tyr Asn Glu Gln Arg Leu Val Arg Glu Ala Asn Asn Cys Ser Ser
        115                 120                 125

CCG GCT GGG ATC CTG GTT GAC GCC ATG TCC CAG AAG CAC CTT CAG ATC    432
Pro Ala Gly Ile Leu Val Asp Ala Met Ser Gln Lys His Leu Gln Ile
    130                 135                 140

AAC CAG ACA TTT GAG GAG CTG CGA CTG GTC ACG CAG GAC ACA GAG AAT    480
Asn Gln Thr Phe Glu Glu Leu Arg Leu Val Thr Gln Asp Thr Glu Asn
145                 150                 155                 160

GAG CTG AAG AAA CTG CAG CAG ACT CAG GAG TAC TTC ATC ATC CAG TAC    528
Glu Leu Lys Lys Leu Gln Gln Thr Gln Glu Tyr Phe Ile Ile Gln Tyr
                165                 170                 175

CAG GAG AGC CTG AGG ATC CAA GCT CAG TTT GCC CAG CTG GCC CAG CTG    576
Gln Glu Ser Leu Arg Ile Gln Ala Gln Phe Ala Gln Leu Ala Gln Leu
            180                 185                 190

AGC CCC CAG GAG CGT CTG AGC CGG GAG ACG GCC CTC CAG CAG AAG CAG    624
Ser Pro Gln Glu Arg Leu Ser Arg Glu Thr Ala Leu Gln Gln Lys Gln
        195                 200                 205

GTG TCT CTG GAG GCC TGG TTG CAG CGT GAG GCA CAG ACA CTG CAG CAG    672
Val Ser Leu Glu Ala Trp Leu Gln Arg Glu Ala Gln Thr Leu Gln Gln
    210                 215                 220

TAC CGC GTG GAG CTG GCC GAG AAG CAC CAG AAG ACC CTG CAG CTG CTG    720
Tyr Arg Val Glu Leu Ala Glu Lys His Gln Lys Thr Leu Gln Leu Leu
225                 230                 235                 240

CGG AAG CAG CAG ACC ATC ATC CTG GAT GAC GAG CTG ATC CAG TGG AAG    768
Arg Lys Gln Gln Thr Ile Ile Leu Asp Asp Glu Leu Ile Gln Trp Lys
                245                 250                 255

CGG CGG CAG CAG CTG GCC GGG AAC GGC GGG CCC CCC GAG GGC AGC CTG    816
Arg Arg Gln Gln Leu Ala Gly Asn Gly Gly Pro Pro Glu Gly Ser Leu
            260                 265                 270

GAC GTG CTA CAG TCC TGG TGT GAG AAG TTG GCC GAG ATC ATC TGG CAG    864
Asp Val Leu Gln Ser Trp Cys Glu Lys Leu Ala Glu Ile Ile Trp Gln
        275                 280                 285
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AAC CGG CAG CAG ATC CGC AGG GCT GAG CAC CTC TGC CAG CAG CTG CCC   912
Asn Arg Gln Gln Ile Arg Arg Ala Glu His Leu Cys Gln Gln Leu Pro
    290                 295                 300

ATC CCC GGC CCA GTG GAG GAG ATG CTG GCC GAG GTC AAC GCC ACC ATC   960
Ile Pro Gly Pro Val Glu Glu Met Leu Ala Glu Val Asn Ala Thr Ile
305                 310                 315                 320

ACG GAC ATT ATC TCA GCC CTG GTG ACC AGC ACA TTC ATC ATT GAG AAG  1008
Thr Asp Ile Ile Ser Ala Leu Val Thr Ser Thr Phe Ile Ile Glu Lys
                325                 330                 335

CAG CCT CCT CAG GTC CTG AAG ACC CAG ACC AAG TTT GCA GCC ACC GTA  1056
Gln Pro Pro Gln Val Leu Lys Thr Gln Thr Lys Phe Ala Ala Thr Val
            340                 345                 350

CGC CTG CTG GTG GGC GGG AAG CTG AAC GTG CAC ATG AAT CCC CCC CAG  1104
Arg Leu Leu Val Gly Gly Lys Leu Asn Val His Met Asn Pro Pro Gln
        355                 360                 365

GTG AAG GCC ACC ATC ATC AGT GAG CAG CAG GCC AAG TCT CTG CTT AAA  1152
Val Lys Ala Thr Ile Ile Ser Glu Gln Gln Ala Lys Ser Leu Leu Lys
    370                 375                 380

AAT GAG AAC ACC CGC AAC GAG TGC AGT GGT GAG ATC CTG AAC AAC TGC  1200
Asn Glu Asn Thr Arg Asn Glu Cys Ser Gly Glu Ile Leu Asn Asn Cys
385                 390                 395                 400

TGC GTG ATG GAG TAC CAC CAA GCC ACG GGC ACC CTC AGT GCC CAC TTC  1248
Cys Val Met Glu Tyr His Gln Ala Thr Gly Thr Leu Ser Ala His Phe
                405                 410                 415

AGG AAC ATG TCA CTG AAG AGG ATC AAG CGT GCT GAC CGG CGG GGT GCA  1296
Arg Asn Met Ser Leu Lys Arg Ile Lys Arg Ala Asp Arg Arg Gly Ala
            420                 425                 430

GAG TCC GTG ACA GAG GAG AAG TTC ACA GTC CTG TTT GAG TCT CAG TTC  1344
Glu Ser Val Thr Glu Glu Lys Phe Thr Val Leu Phe Glu Ser Gln Phe
        435                 440                 445

AGT GTT GGC AGC AAT GAG CTT GTG TTC CAG GTG AAG ACT CTG TCC CTA  1392
Ser Val Gly Ser Asn Glu Leu Val Phe Gln Val Lys Thr Leu Ser Leu
    450                 455                 460

CCT GTG GTT GTC ATC GTC CAC GGC AGC CAG GAC CAC AAT GCC ACG GCT  1440
Pro Val Val Val Ile Val His Gly Ser Gln Asp His Asn Ala Thr Ala
465                 470                 475                 480

ACT GTG CTG TGG GAC AAT GCC TTT GCT GAG CCG GGC AGG GTG CCA TTT  1488
Thr Val Leu Trp Asp Asn Ala Phe Ala Glu Pro Gly Arg Val Pro Phe
                485                 490                 495

GCC GTG CCT GAC AAA GTG CTG TGG CCG CAG CTG TGT GAG GCG CTC AAC  1536
Ala Val Pro Asp Lys Val Leu Trp Pro Gln Leu Cys Glu Ala Leu Asn
            500                 505                 510

ATG AAA TTC AAG GCC GAA GTG CAG AGC AAC CGG GGC CTG ACC AAG GAG  1584
Met Lys Phe Lys Ala Glu Val Gln Ser Asn Arg Gly Leu Thr Lys Glu
        515                 520                 525

AAC CTC GTG TTC CTG GCG CAG AAA CTG TTC AAC AAC AGC AGC AGC CAC  1632
Asn Leu Val Phe Leu Ala Gln Lys Leu Phe Asn Asn Ser Ser Ser His
    530                 535                 540

CTG GAG GAC TAC AGT GGC CTG TCC GTG TCC TGG TCC CAG TTC AAC AGG  1680
Leu Glu Asp Tyr Ser Gly Leu Ser Val Ser Trp Ser Gln Phe Asn Arg
545                 550                 555                 560
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GAG AAC TTG CCG GGC TGG AAC TAC ACC TTC TGG CAG TGG TTT GAC GGG  1728
Glu Asn Leu Pro Gly Trp Asn Tyr Thr Phe Trp Gln Trp Phe Asp Gly
                565                 570                 575

GTG ATG GAG GTG TTG AAG AAG CAC CAC AAG CCC CAC TGG AAT GAT GGG  1776
Val Met Glu Val Leu Lys Lys His His Lys Pro His Trp Asn Asp Gly
            580                 585                 590

GCC ATC CTA GGT TTT GTG AAT AAG CAA CAG GCC CAC GAC CTG CTC ATC  1824
Ala Ile Leu Gly Phe Val Asn Lys Gln Gln Ala His Asp Leu Leu Ile
        595                 600                 605

AAC AAG CCC GAC GGG ACC TTC TTG TTG CGC TTT AGT GAC TCA GAA ATC  1872
Asn Lys Pro Asp Gly Thr Phe Leu Leu Arg Phe Ser Asp Ser Glu Ile
    610                 615                 620

GGG GGC ATC ACC ATC GCC TGG AAG TTT GAC TCC CCG GAA CGC AAC CTG  1920
Gly Gly Ile Thr Ile Ala Trp Lys Phe Asp Ser Pro Glu Arg Asn Leu
625                 630                 635                 640

TGG AAC CTG AAA CCA TTC ACC ACG CGG GAT TTC TCC ATC AGG TCC CTG  1968
Trp Asn Leu Lys Pro Phe Thr Thr Arg Asp Phe Ser Ile Arg Ser Leu
                645                 650                 655

GCT GAC CGG CTG GGG GAC CTG AGC TAT CTC ATC TAT GTG TTT CCT GAC  2016
Ala Asp Arg Leu Gly Asp Leu Ser Tyr Leu Ile Tyr Val Phe Pro Asp
            660                 665                 670

CGC CCC AAG GAT GAG GTC TTC TCC AAG TAC TAC ACT CCT GTG CTG GCT  2064
Arg Pro Lys Asp Glu Val Phe Ser Lys Tyr Tyr Thr Pro Val Leu Ala
        675                 680                 685

AAA GCT GTT GAT GGA TAT GTG AAA CCA CAG ATC AAG CAA GTG GTC CCT  2112
Lys Ala Val Asp Gly Tyr Val Lys Pro Gln Ile Lys Gln Val Val Pro
    690                 695                 700

GAG TTT GTG AAT GCA TCT GCA GAT GCT GGG GGC AGC AGC GCC ACG TAC  2160
Glu Phe Val Asn Ala Ser Ala Asp Ala Gly Gly Ser Ser Ala Thr Tyr
705                 710                 715                 720

ATG GAC CAG GCC CCC TCC CCA GCT GTG TGC CCC CAG GCT CCC TAT AAC  2208
Met Asp Gln Ala Pro Ser Pro Ala Val Cys Pro Gln Ala Pro Tyr Asn
                725                 730                 735

ATG TAC CCA CAG AAC CCT GAC CAT GTA CTC GAT CAG GAT GGA GAA TTC  2256
Met Tyr Pro Gln Asn Pro Asp His Val Leu Asp Gln Asp Gly Glu Phe
            740                 745                 750

GAC CTG GAT GAG ACC ATG GAT GTG GCC AGG CAC GTG GAG GAA CTC TTA  2304
Asp Leu Asp Glu Thr Met Asp Val Ala Arg His Val Glu Glu Leu Leu
        755                 760                 765

CGC CGA CCA ATG GAC AGT CTT GAC TCC CGC CTC TCG CCC CCT GCC GGT  2352
Arg Arg Pro Met Asp Ser Leu Asp Ser Arg Leu Ser Pro Pro Ala Gly
    770                 775                 780

CTT TTC ACC TCT GCC AGA GGC TCC CTC TCA TGA                      2385
Leu Phe Thr Ser Ala Arg Gly Ser Leu Ser
785                 790
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 794 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Gly Trp Ile Gln Ala Gln Gln Leu Gln Gly Asp Ala Leu Arg
 1               5                  10                  15

Gln Met Gln Val Leu Tyr Gly Gln His Phe Pro Ile Glu Val Arg His
            20                  25                  30

Tyr Leu Ala Gln Trp Ile Glu Ser Gln Pro Trp Asp Ala Ile Asp Leu
        35                  40                  45

Asp Asn Pro Gln Asp Arg Ala Gln Ala Thr Gln Leu Leu Glu Gly Leu
    50                  55                  60

Val Gln Glu Leu Gln Lys Lys Ala Glu His Gln Val Gly Glu Asp Gly
65                  70                  75                  80

Phe Leu Leu Lys Ile Lys Leu Gly His Tyr Ala Thr Gln Leu Gln Lys
                85                  90                  95

Thr Tyr Asp Arg Cys Pro Leu Glu Leu Val Arg Cys Ile Arg His Ile
            100                 105                 110

Leu Tyr Asn Glu Gln Arg Leu Val Arg Glu Ala Asn Asn Cys Ser Ser
        115                 120                 125

Pro Ala Gly Ile Leu Val Asp Ala Met Ser Gln Lys His Leu Gln Ile
    130                 135                 140

Asn Gln Thr Phe Glu Glu Leu Arg Leu Val Thr Gln Asp Thr Glu Asn
145                 150                 155                 160

Glu Leu Lys Lys Leu Gln Gln Thr Gln Glu Tyr Phe Ile Ile Gln Tyr
                165                 170                 175

Gln Glu Ser Leu Arg Ile Gln Ala Gln Phe Ala Gln Leu Ala Gln Leu
            180                 185                 190

Ser Pro Gln Glu Arg Leu Ser Arg Glu Thr Ala Leu Gln Gln Lys Gln
        195                 200                 205

Val Ser Leu Glu Ala Trp Leu Gln Arg Glu Ala Gln Thr Leu Gln Gln
    210                 215                 220

Tyr Arg Val Glu Leu Ala Glu Lys His Gln Lys Thr Leu Gln Leu Leu
225                 230                 235                 240

Arg Lys Gln Gln Thr Ile Ile Leu Asp Asp Glu Leu Ile Gln Trp Lys
                245                 250                 255

Arg Arg Gln Gln Leu Ala Gly Asn Gly Gly Pro Pro Glu Gly Ser Leu
            260                 265                 270

Asp Val Leu Gln Ser Trp Cys Glu Lys Leu Ala Glu Ile Ile Trp Gln
        275                 280                 285

Asn Arg Gln Gln Ile Arg Arg Ala Glu His Leu Cys Gln Gln Leu Pro
    290                 295                 300

Ile Pro Gly Pro Val Glu Glu Met Leu Ala Glu Val Asn Ala Thr Ile
305                 310                 315                 320

Thr Asp Ile Ile Ser Ala Leu Val Thr Ser Thr Phe Ile Ile Glu Lys
                325                 330                 335
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Gln Pro Pro Gln Val Leu Lys Thr Gln Thr Lys Phe Ala Ala Thr Val
            340                 345                 350

Arg Leu Leu Val Gly Gly Lys Leu Asn Val His Met Asn Pro Pro Gln
        355                 360                 365

Val Lys Ala Thr Ile Ile Ser Glu Gln Gln Ala Lys Ser Leu Leu Lys
    370                 375                 380

Asn Glu Asn Thr Arg Asn Glu Cys Ser Gly Glu Ile Leu Asn Asn Cys
385                 390                 395                 400

Cys Val Met Glu Tyr His Gln Ala Thr Gly Thr Leu Ser Ala His Phe
                405                 410                 415

Arg Asn Met Ser Leu Lys Arg Ile Lys Arg Ala Asp Arg Arg Gly Ala
            420                 425                 430

Glu Ser Val Thr Glu Glu Lys Phe Thr Val Leu Phe Glu Ser Gln Phe
        435                 440                 445

Ser Val Gly Ser Asn Glu Leu Val Phe Gln Val Lys Thr Leu Ser Leu
    450                 455                 460

Pro Val Val Val Ile Val His Gly Ser Gln Asp His Asn Ala Thr Ala
465                 470                 475                 480

Thr Val Leu Trp Asp Asn Ala Phe Ala Glu Pro Gly Arg Val Pro Phe
                485                 490                 495

Ala Val Pro Asp Lys Val Leu Trp Pro Gln Leu Cys Glu Ala Leu Asn
            500                 505                 510

Met Lys Phe Lys Ala Glu Val Gln Ser Asn Arg Gly Leu Thr Lys Glu
        515                 520                 525

Asn Leu Val Phe Leu Ala Gln Lys Leu Phe Asn Asn Ser Ser Ser His
    530                 535                 540

Leu Glu Asp Tyr Ser Gly Leu Ser Val Ser Trp Ser Gln Phe Asn Arg
545                 550                 555                 560

Glu Asn Leu Pro Gly Trp Asn Tyr Thr Phe Trp Gln Trp Phe Asp Gly
                565                 570                 575

Val Met Glu Val Leu Lys Lys His His Lys Pro His Trp Asn Asp Gly
            580                 585                 590

Ala Ile Leu Gly Phe Val Asn Lys Gln Gln Ala His Asp Leu Leu Ile
        595                 600                 605

Asn Lys Pro Asp Gly Thr Phe Leu Leu Arg Phe Ser Asp Ser Glu Ile
    610                 615                 620

Gly Gly Ile Thr Ile Ala Trp Lys Phe Asp Ser Pro Glu Arg Asn Leu
625                 630                 635                 640

Trp Asn Leu Lys Pro Phe Thr Thr Arg Asp Phe Ser Ile Arg Ser Leu
                645                 650                 655

Ala Asp Arg Leu Gly Asp Leu Ser Tyr Leu Ile Tyr Val Phe Pro Asp
            660                 665                 670

Arg Pro Lys Asp Glu Val Phe Ser Lys Tyr Tyr Thr Pro Val Leu Ala
        675                 680                 685
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Lys Ala Val Asp Gly Tyr Val Lys Pro Gln Ile Lys Gln Val Val Pro
    690                 695                 700

Glu Phe Val Asn Ala Ser Ala Asp Ala Gly Gly Ser Ser Ala Thr Tyr
705                 710                 715                 720

Met Asp Gln Ala Pro Ser Pro Ala Val Cys Pro Gln Ala Pro Tyr Asn
                725                 730                 735

Met Tyr Pro Gln Asn Pro Asp His Val Leu Asp Gln Asp Gly Glu Phe
            740                 745                 750

Asp Leu Asp Glu Thr Met Asp Val Ala Arg His Val Glu Glu Leu Leu
        755                 760                 765

Arg Arg Pro Met Asp Ser Leu Asp Ser Arg Leu Ser Pro Pro Ala Gly
    770                 775                 780

Leu Phe Thr Ser Ala Arg Gly Ser Leu Ser
785                 790
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATTTCCCA GAAAAGGAAC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTATCACCCA GTCAAGGAAC 20

WHAT IS CLAIMED IS:

1. An isolated human signal transducer and activator of transcription 5 (hStat 5, SEQUENCE ID NO:2), or a fragment thereof having an hStat 5-specific binding affinity.

2. Human Stat 5 or an hStat 5 fragment according to claim 1 comprising at least one of amino acid sequence ATQLQK (SEQUENCE ID NO:2, residues 91-96); YDRCPLELV (SEQUENCE ID NO:2, residues 98-106); NNCSSP (SEQUENCE ID NO:2, residues 124-129); QQLAGNGG (SEQUENCE ID NO:2, residues 259-266); NGGPPEG (SEQUENCE ID NO:2, residues 264-270); NASADAG (SEQUENCE ID NO:2, residues 708-714); SATYMDQAP (SEQUENCE ID NO:2, residues 717-725); SPAVCPQAP (SEQUENCE ID NO:2, residues 726-734); YNMYPQNPD (SEQUENCE ID NO:2, residues 735-743); HVLDQDGEF (SEQUENCE ID NO:2, residues 744-752); DLDETMDVA (SEQUENCE ID NO:2, residues 753-761); VEELLRR (SEQUENCE ID NO:2, residues 764-770); MDSLDSRLS (SEQUENCE ID NO:2, residues 772-780); and FTSARGSLS (SEQUENCE ID NO:2, residues 786-794).

3. An isolated antibody that selectively binds hStat 5 according to claim 1.

4. An isolated nucleic acid encoding hStat 5 or an hStat 5 fragment according to claim 1.

5. A vector comprising a nucleic acid according to claim 4 operably linked to a transcription regulatory element, said nucleic acid being joined in sequence directly to a nucleotide not naturally joined to said nucleic acid.

6. A cell comprising a nucleic acid according to claim 4, said nucleic acid being joined in sequence directly to a nucleotide not naturally joined to said nucleic acid.

7. A method of identifying an agent useful in the diagnosis or treatment of disease associated with the binding of hStat 5 to an intracellular hStat 5 binding target, said method comprising the steps of:

forming a first mixture comprising hStat 5 or an hStat 5 fragment according to claim 1, an intracellular hStat 5 binding target, and a prospective agent at a first concentration;

forming a second mixture comprising said hStat 5 or hStat 5 fragment, said intracellular hStat 5 binding target, and said prospective agent at a second concentration different from said first concentration;

incubating said first and second mixtures;

measuring the binding in each mixture of said intracellular hStat 5 binding target to said hStat 5 or hStat 5 fragment;

wherein a difference in said binding in said first and second mixtures indicates said agent is capable of modulating the binding of hStat 5 or an hStat 5 fragment to an intracellular hStat 5 binding target.

8. A method of identifying an hStat 5 selective agent, said method comprising the steps of:

forming a mixture of a prospective agent and hStat 5 or an hStat 5 fragment according to claim 1;

incubating said mixture;

identifying specifically bound complexes of said hStat 5 or hStat 5 fragment and said agent;

wherein the presence of said complexes indicates said agent is a hStat 5-selective agent.

9. A method according to claim 7 wherein said first mixture comprises a first fusion protein comprising an hStat 5 fragment fused to one of the transcription activation or the DNA binding domain of a transription factor and a second fusion protein comprising an intracellular hStat 5 binding target fused to the other of the transcription activation or the DNA binding domain of a transcription factor.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 01/68; G01N 33/53; C07K 14/47
US CL :435/6, 7.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG,
search terms: human, STAT5, mammary gland factor, assay?, bind?, agent?, drug?, modulat?, mixture?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WAKAO et al. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. The EMBO Journal. 01 May 1994, Vol. 13, No. 9, pages 2182-2191, see entire document.	1-2, 7-9
A, P	AZAM et al. Interleukin-3 signals through multiple isoforms of Stat5. The EMBO Journal. 03 April 1995, Vol. 14, No. 7, pages 1402-1411, see entire document.	1-2, 7-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
13 MAY 1996

Date of mailing of the international search report
31 MAY 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
TERRY A. MCKELVEY
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02457

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2 and 7-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02457

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions o. groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-2 and 7-9, drawn to STAT proteins and assay using STAT protein.

Group II, claim 3, drawn to antibody that binds STAT protein.

Group III, claims 4-6, drawn to nucleic acid, vector and cell which contains the STAT gene.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the STAT protein of Group I, the antibody that binds to STAT protein of Group II and the nucleic acid, vector and cell containing STAT gene of Group III are physically, chemically and biologically different and distinct from each other and thus have no special technical feature in common. The antibody of Group II and the nucleic acid, vector and cell of Group III are not used in the method of Group I and thus there is no common special technical feature. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.